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Cell Cycle-related and Endogenously Controlled Circadian Photosynthetic Rhythms in *Euglena*[1]

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DANIELLE L. LAVAL-MARTIN[2], DAVID J. SHUCH, AND LELAND N. EDMUNDS, JR.[3]
Division of Biological Sciences, State University of New York, Stony Brook, New York 11794

ABSTRACT

The data presented for three strains of *Euglena gracilis* corroborate previous reports of a diel rhythm in photosynthetic capacity in division-synchronized cultures of this alga and extend these studies to free running, dividing and nondividing (stationary) cultures maintained in either 24-hour or 40-minute cycles of light and darkness. During synchronous growth entrained by LD: 12,12 or free running under LD: 1/3,1/3, photosynthetic CO_2 fixation was rhythmic with a period (24.0 or about 30 hours) corresponding to the period of the cell division rhythm in the population. Furthermore, the rhythm in CO_2 fixation (per cell) found in nondividing cultures maintained in LD: 12,12 persisted in LD: 1/3,1/3 for weeks with a free running, circadian period of approximately 30 hours. An endogenous, circadian rhythm in cellular chlorophyll was found to exist, independently of cell division, under both light regimens and in each individual experiment; this observation could reflect changes in the functional role of the pigment. In cultures maintained in LD: 1/3,1/3, the phase relationship between the rhythm of photosynthetic capacity and that of chlorophyll content varied, suggesting the possibility of desynchronization among circadian rhythms in a multioscillator, unicellular organism.

Circadian rhythms in photosynthesis have been observed in a number of higher plants as well as in algae (16). Several studies have reported a rhythm of photosynthetic capacity during the cell cycle of the alga flagellate *Euglena* (3, 4, 22, 32), although the results are often conflicting. Walther and Edmunds (32) demonstrated a clear 24-h rhythm in the capacity of *Euglena* to fix CO_2 in cultures synchronized by appropriately chosen light (12,000 lux) cycles (LD: 10,14).[4] This rhythm was observed in both the exponential growth phase as well as in nondividing cells in the stationary phase. Attempts to demonstrate a persisting circadian rhythm under dim LL (750 lux) or higher frequency LD cycles [LD: 2,4 (12,000 lux)] were not successful.

This lack of long term persistence of the photosynthetic capacity rhythm and hence the inability to conclude that an endogenous circadian clock underlies the overt periodicity, could have been due to the nature of the light conditions chosen. The 750 lux-LL utilized completely suppressed cell division and, by implication, perturbed the over-all physiology of the cell. This was reflected in the greatly decreased rate of CO_2 fixation observed, making the assay of the rhythm virtually impossible. Higher intensities of illumination could not be used, since circadian rhythms often damp out under such conditions (30), and DD could hardly be employed in a phototrophic system. The attempt to circumvent this quandary by imposing a 6-h light-dark cycle (LD: 2,4) was open to a similar shortcoming since the cells received only 8 h of light (12,000 lux) in a 24-h time span (in contrast to the 10 h afforded by the synchronizing LD: 10,14 regime). In some instances cultures exhibited direct entrainment to this shorter period (32).

We have monitored photosynthetic capacity and Chl content in three strains of *Euglena gracilis* synchronized by a LD: 12,12 (7,000 lux) cycle during both the exponential growth and stationary phases and then released into a LD: 1/3,1/3 (7,000 lux) regimen. This particular higher frequency, 40-min cycle was selected so as to afford an amount of light during a 24-h time span identical to that received in the LD: 12,12 cycle. Direct entrainment of the cultures to a 40-min period was not to be expected; the cell division rhythm is known to persist with a circadian period under these conditions (13, 14).

Lonergan and Sargent (20) have demonstrated a persisting circadian rhythm of photosynthetic O_2 evolution in *Euglena* during both the exponential growth and stationary phases under dim illumination but have questioned whether CO_2 fixation was rhythmic during the exponential growth phase. Inasmuch as it would seem improbable to have a dissociation of the O_2 and CO_2 photosynthetic rhythms, a detailed investigation of CO_2 fixation in *Euglena* further appears warranted.

MATERIALS AND METHODS

Organisms and Culture Conditions. Three strains of *E. gracilis* Klebs were utilized, all derived from an earlier "Z strain": C438, maintained in this laboratory since 1965; Z-Gif, maintained for over 10 years at Gif-sur-Yvette, and a DCMU-resistant strain, EgZR, grown for 3 years in the presence of 25 μM DCMU in a medium (pH 3.5) containing 33 mM of DL-lactate as the sole utilizable carbon source (1, 19).

The two similar wild type strains were grown photoautotrophically in a modified Cramer and Myers' (5) medium supplemented with a 3-fold higher concentration of vitamins B_1 and B_{12} than previously described (14). For the EgZR strain, 1 ml of 25 mM DCMU in isopropyl alcohol was added per liter of autoclaved medium. Magnetically stirred, aerated (575–625 ml min^{-1}), batch cultures (4-, 8-, or 16-liter) were maintained at 25 C in environmental chambers furnished with clock-programed, cool-white fluorescent lights (7,000 lux incident illumination) as described previously (32). Cultures were synchronized by imposed LD: 12,12 light cycles. Cell number was monitored every 2 h by a miniatur-

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[2] Present address: Laboratoire de Cytophysiologie de la Photosynthèse, CNRS, 91190 Gif-sur-Yvette, France.

[3] Author to whom reprint requests should be sent. Present address: Laboratoire du Phytotron, CNRS, 91190 Gif-sur-Yvette, France.

[4] Abbreviations: a repetitive cycle of x hours of light and y hours of darkness will be denoted by LD: x,y (where the period [T] of the imposed cycle = [x + y]); LL: continuous light; DD: continuous darkness; T: period of LD cycle; τ: period of overt biological rhythm; PC: photosynthetic capacity; PE: photosynthetic efficacy (maximal rate of CO_2 fixation per milligram of chlorophyll under saturating light).

ized fraction collector and automatic pipetting device (11) and a Coulter Counter model B electronic particle counter (Coulter Electronics, Hialeah, Fla.).

Chl Determination. Chl *a* and *b* were analyzed (model 2400-2 Gilford recording spectrophotometer, Gilford Instrument Laboratories, Oberlin, Ohio) in an acetone extract according to MacKinney (23). Aliquots of culture, varying from 200 to 10 ml (according to culture age and cell titer), were centrifuged at 6,000*g* for 10 min. The pellet, resuspended in Tris (0.05 M, pH 7.5), was extracted with four times its volume of acetone, vigorously stirred with $CaCO_3$, and centrifuged again (12,000*g*, 10 min). In all determinations, the initial culture aliquot, the volume of the resuspended pellet, and the amount of acetone added were chosen so as to give *A* at 645 and 663 nm ranging between 0.2 to 0.5 and 0.6 to 1.5, respectively.

In order to measure Chl *b* more accurately, a kinetic method of determination (6, 7) was used which is based on the controlled pheophytinization of Chl *a* and Chl *b*. In a 90:10 (v/v) acetone-water pigment extract, HCl causes a 30-fold more rapid transformation of Chl *a* into pheophytin *a* than of Chl *b* into pheophytin *b*. The extrapolation on semilogarithmic paper (7) of the exponential curve recorded at 645 nm enables one to determine the decay in *A* ($\Delta A_{b,645\ nm}$) accompanying the pheophytinization of Chl *b* into pheophytin *b*. Under these conditions, Chl *b* ($\mu g\ ml^{-1}$) = 36 $\Delta A_{b,645\ nm}$, and total Chl ($\mu g\ ml^{-1}$) = 7.58 $A_{663\ nm}$ + 18.7 $A_{645\ nm}$.

Photosynthetic Fixation of $^{14}CO_2$. Culture aliquots of 2 ml were taken at 3-h intervals and held at 27 C for 15 min in a water bath. Thereupon, 500 μl of sodium bicarbonate solution (40 mM in Tris [250 mM, pH 8.3]), labeled with [^{14}C]sodium bicarbonate to give a specific radioactivity of 0.17 to 0.34 mCi/mmol, was added after 5 min of preillumination to yield 20 μmol of bicarbonate in the final reaction mixture. The reaction was stopped after 10 min in either saturating light (28,000 lux) or the dark by adding 0.5 ml of glacial acetic acid (17.4 N). The contents of the test tube, followed by three rinses with 3.5 N diluted glacial acetic acid, were collected on Millipore filters that were then dried, placed in scintillation fluid (Amersham/Searle) and counted in a Packard Tri-Carb liquid scintillation spectrometer (model 3320). Results were expressed as mol CO_2 fixed h^{-1} $(10^6\ cells)^{-1}$ (photosynthetic capacity [PC]) or mol CO_2 fixed h^{-1} (mg Chl)$^{-1}$ (photosynthetic efficacy [PE]) with reference to a standard prepared from the labeled 400 mM bicarbonate diluted 10-fold with 0.2 N NaOH. (Depending on the experiment, the standard varied between 1.5 and 3.0 $\times$ 10^5 cpm [μmol CO_2]$^{-1}$.)

RESULTS

Our experimental protocol was directed toward two distinct but interrelated goals: to elucidate the relationship between photosynthetic rhythms and the cell division cycle in synchronized cultures of *Euglena*, and to examine possible persisting circadian rhythms of photosynthesis in nondividing cultures that were no longer under the entraining influence of 24-h light-dark cycles. The situation is complicated by the fact that synchronously *dividing* cultures, previously entrained by diel light cycles, may be themselves free running under certain conditions, such as constant dim illumination or high frequency LD cycles (13, 14); hence, they might be expected also to evidence free running (and presumably, but not necessarily causally, cell division cycle-related) photosynthetic rhythms. For the reasons discussed previously, we decided to replace the classical conditions of continuous dim illumination normally used to elicit free running photosynthetic rhythms (20, 32) with a high frequency LD: 1/3,1/3 cycle. This regime was imposed at two different phases of population growth: (*a*) on essentially nondividing Z or Z_R strains that had reached the stationary phase (infradian growth mode) after having been division-synchronized by LD: 12,12 throughout the exponential growth phase; and (*b*) on synchronously dividing wild Z strains still in the exponential growth phase that had been entrained by several prior LD: 12,12 cycles. In the latter case, both the cell division rhythm and the several photosynthetic rhythms could be monitored under the imposed high frequency cycle until the cultures attained the infradian growth mode, whereupon only the photosynthetic rhythms were followed during ensuing weeks.

For each of these cultures the PC of the cells to fix CO_2 under saturating light conditions, Chl (expressed as μg Chl/10^6 cells), and the PE of the cells were monitored (usually at 3-h intervals). The results obtained under both light-dark regimens, imposed at either phase of population increase, follow.

24-h Light-Dark Cycles: LD: 12,12 (T = 24 h). An investigation was undertaken during the synchronous growth cycle of two wild type *Euglena* Z strains (Stony Brook and Gif-sur-Yvette) according to the following protocol. Two identical (same initial inoculum) back-to-back cultures were grown synchronously in LD: 12,12 cycles that were 180° out of phase (*i.e.* L = 0900–2100 or 2100–0900 local time). Photosynthetic activity (PC, PE, Chl) was monitored at 3-h intervals in the parallel cultures. In order to standardize the data thus obtained, the results of the parallel runs for each strain are expressed as per cent of the maximal response found in cultures sampled over a 2-day time span. Mean values at any given sampling time during the LD cycle, together with the standard errors of the means (4–10 experimental values for each point) have been calculated (Fig. 1).

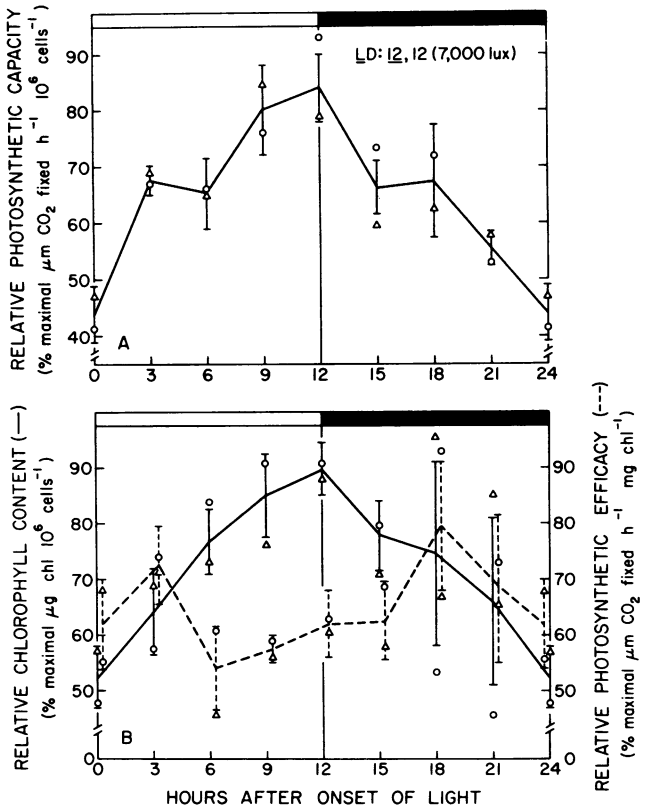

FIG. 1. Statistical composite plot of the rhythms of (A) PC and (B) cellular Chl content (——) and PE (- - -) in two different wild type strains (Stony Brook [○] and Gif-sur-Yvette [△]) of synchronously dividing batch cultures of *E. gracilis* (Z) entrained by a LD: 12,12 (7,000 lux) cycle at 25 C. Measurements were made throughout 2 consecutive days for each strain using back-to-back cultures 180° out of phase. The data were pooled and expressed as per cent of the maximal value found during the 48-h experiments. The number of data for each h varied between 4 and 10. Curves are drawn through the mean values; vertical bars indicate twice the standard errors of the means. Absolute maximal values for CO_2 fixation, total cellular Chl content, and PE were, respectively: 8.78 μmol CO_2 fixed h^{-1} 10^{-6} cells^{-1}, 25.17 μg Chl 10^{-6} cells^{-1}, and 685 μmol CO_2 h^{-1} mg^{-1} Chl. The activities of the two strains were comparable.

It has been demonstrated repeatedly (10, 11) that cell division occurs primarily during the first 6 to 8 h of the 12-h dark period, whereas cell number remains constant throughout the light periods. In these back-to-back cultures the step sizes (ss), or factorial increases in cell concentration, of the synchronous fission bursts were about 1.5, but there was a doubling in amplitude of the composite curve of PC with a maximum value occurring at the end of the light period and with two secondary peaks (or shoulders) appearing at the 3rd h of the light period and usually at the 6th h of darkness (when the frequency of sampling permitted its detection). We regard these secondary peaks as significant considering the fact that they were almost always present (with varying amplitude in individual experiments) in both back-to-back (Fig. 1) and long term cultures of both the Z and the DCMU-adapted Z_R strains (see Figs. 2 and 3, panel 1, respectively). A rhythm in total cellular Chl content exists also (Fig. 1, lower panel). During the light, relative Chl content increases by a factor of 1.7; then, during the dark, it diminishes back to its initial value. This reduction in Chl/cell does not occur at a linear rate; rather, it decreases quite rapidly for the first 3 h of darkness and then diminishes at a slower rate—just the opposite from the rate of cell division in the population (11). These findings thus suggest that the mechanisms underlying the diminution of pigment content may be operating independently of cell division controls *per se* and any simple partitioning of products among the daughter cells. Finally, there appears to be a rhythm in PE during the LD cycle (Fig. 1, lower panel, dashed line) which exhibits two peaks, one at the beginning of L and the other in the middle of D (although the standard error is rather large in this latter case). Both peaks occur when the concentration of Chl per cell is approximately 70 to 75% (or even less) of the maximal value attained at the end of L.

The foregoing results for synchronously dividing cultures obtained over a 2-day time span were corroborated by a more extended analysis of not only the Z strain (Stony Brook) (Fig. 2, left column) but also the DCMU-adapted EgZR strain (Z_R, Fig. 3, left column). Sampling took place between the 3rd and 5th (Z) or the 5th and 7th (Z_R) days of culture growth. The step sizes (ss) of the two synchronous division bursts that took place in the Z and Z_R cultures were 1.93 and 1.84, and 1.87 and 1.63, respectively, indicating that the populations approximately doubled after each burst had occurred. The amount of CO_2 fixed per cell per unit time (PC) varied in both strains by a factor of 2 to 3 (Figs. 2 and 3, panel 1) with a major peak occurring somewhere between the mid- and late-light period. This reconfirms earlier data from this laboratory (32). The same order of magnitude is observed among the changes in the total amount of Chl per cell, which exhibits a pronounced decrease during the dark intervals (Figs. 2 and 3, panel 4). This decrement in cellular Chl content during darkness also has been reported heretofore (12). The capacity to fix CO_2 in both the Z and Z_R strains often appears to manifest two peaks during the 12-h light period (*e.g.* Fig. 3, panel 1), whereas both cultures exhibit an apparently linear increase and decrease in cellular Chl content during the light and ensuing dark periods. Although the secondary peak or shoulder was not observed in every light interval, the analysis of all of the available data (Fig. 1A) indicates its existence.

In order to ascertain to what degree the observed cyclic changes in photosynthetic activity could be divorced from the cell division cycle itself, these studies on dividing populations of *Euglena* were subsequently extended to stationary (or, more accurately, very slowly increasing infradian) cultures. Typical results for the Z and Z_R strains are shown in Figures 2 and 3 (middle columns) for the 12th to 14th day after inoculation (*i.e.* some 7 or 5 days, respectively, after the measurements during the exponential growth phase had been completed). Cell titer was 2.0×10^5 or 1.25×10^5 cells/ml, respectively.

The two strains each exhibited clear-cut, 24-h variations in PC (panel 2), total Chl (panel 5), and PE (panel 8) whose profiles were comparable to those observed in doing the synchronous growth cycle (panels 1, 4, and 7). The amplitude of the CO_2 fixation rhythm was greater than that of the change in total cellular Chl, suggesting that the efficiency of photosynthetic mechanisms is itself changing. The diurnal variations of the photosynthetic variables manifested in growing populations can occur in the absence of the driving force of the cell division cycle, substantiating earlier observations by Walther and Edmunds (32).

High Frequency Light-Dark Cycles: LD: 1/3,1/3 (T = 40 min). The final segment of this investigation concerned the behavior of the photosynthetic system in the absence of a driving 24-h LD cycle in stationary cell populations exposed to high frequency cycles of light (20 min) and darkness (20 min). These 40-min cycles were applied to the Z strain beginning at the 11th h of the usual light period (in LD: 12,12) on the 15th day of culture, and to the Z_R strain starting at the 3rd h of darkness (in LD: 12,12) on the 14th day of culture (Figs. 2 and 3, respectively, right columns). Photosynthetic measurements were carried out beginning either the 3rd (Z) or the 5th (Z_R) day after the initiation of the high frequency light regimen to allow any transients to subside and a new steady-state equilibrium to be attained.

Under such rapid alternations of L and D, a cyclic change in PC in the Z strain was observed, increasing by a factor of 8 (Fig. 2, panel 3), although the total Chl content increased by only 25 to 30% (panel 6). This can be attributed to significant changes in the PE of the pigment, as depicted in panel 9. The Z_R culture exhibited the same rhythmicities albeit with lower amplitudes (Fig. 3, panels 3, 6, and 9).

These changes in PE can be correlated with changes in the Chl *a*/Chl *b* ratio as calculated according to MacKinney (23) and plotted in Figures 2 and 3 (panel 6) for the two strains. In general, the increments in total cellular Chl (and PC) beginning with the onset of the subjective light period (where the main light period would have fallen had the LD: 12,12 cycle been continued) are accompanied by a sharp diminution in the Chl *a*/Chl *b* ratio while, simultaneously, total PE increases (panel 9). Conversely, during the subjective night, total Chl (and PC) decrease, the Chl *a*/Chl *b* ratio increases, and PE decreases.

The data presented in Figures 2 and 3 for both the Z and Z_R strains also demonstrate the *persistence* of the circadian rhythms of the photosynthetic parameters monitored (PC, total Chl, and PE) for a minimum of 3 to 5 days in LD: 1/3,1/3; the intervals between the two observed peaks (or troughs) in PC (panel 3) or total Chl (panel 6) for either strain (Figs. 2 and 3) approximate 27 h. Since the data were insufficient, however, to permit a rigorous quantification of the free running period (τ_{FR}), a more detailed statistical study was desirable. Further, the question arose to the behavior of the photosynthetic rhythms in *dividing* cultures exposed to the high frequency LD regimen—conditions under which the cell division cycle itself has been shown to free run (13, 14). Accordingly, we monitored PC, PE, total Chl, and Chl *b* in LD: 1/3,1/3 during *both* the exponential phase of growth and the stationary (infradian) phase in five different cultures of the Z strain (Stony Brook) that had been previously entrained for several days by LD: 12,12 (Fig. 4).

In this figure both the mean values for each variable and their moving 3-point averages are indicated. In order to facilitate comparison the resulting periodicities (which can be considered for all intents and purposes as "free running" under the imposed high frequency LD cycle) have been normalized ($\bar{\tau}_{FR} \simeq 30$ h) to a 24-h time scale. During the exponential phase of growth, a clear rhythm in PC (Fig. 4, upper left) was observed in cultures exposed to LD: 1/3,1/3 with an amplitude comparable to that seen in LD: 12,12 (Fig. 1A). Indeed, this rhythm was even more well defined in each of the five individual experiments upon which this statistical composite plot was based.

The two secondary peaks or shoulders in PC usually observed in LD: 12,12 at the 3rd h of L and the 6th h of D (Fig. 1A),

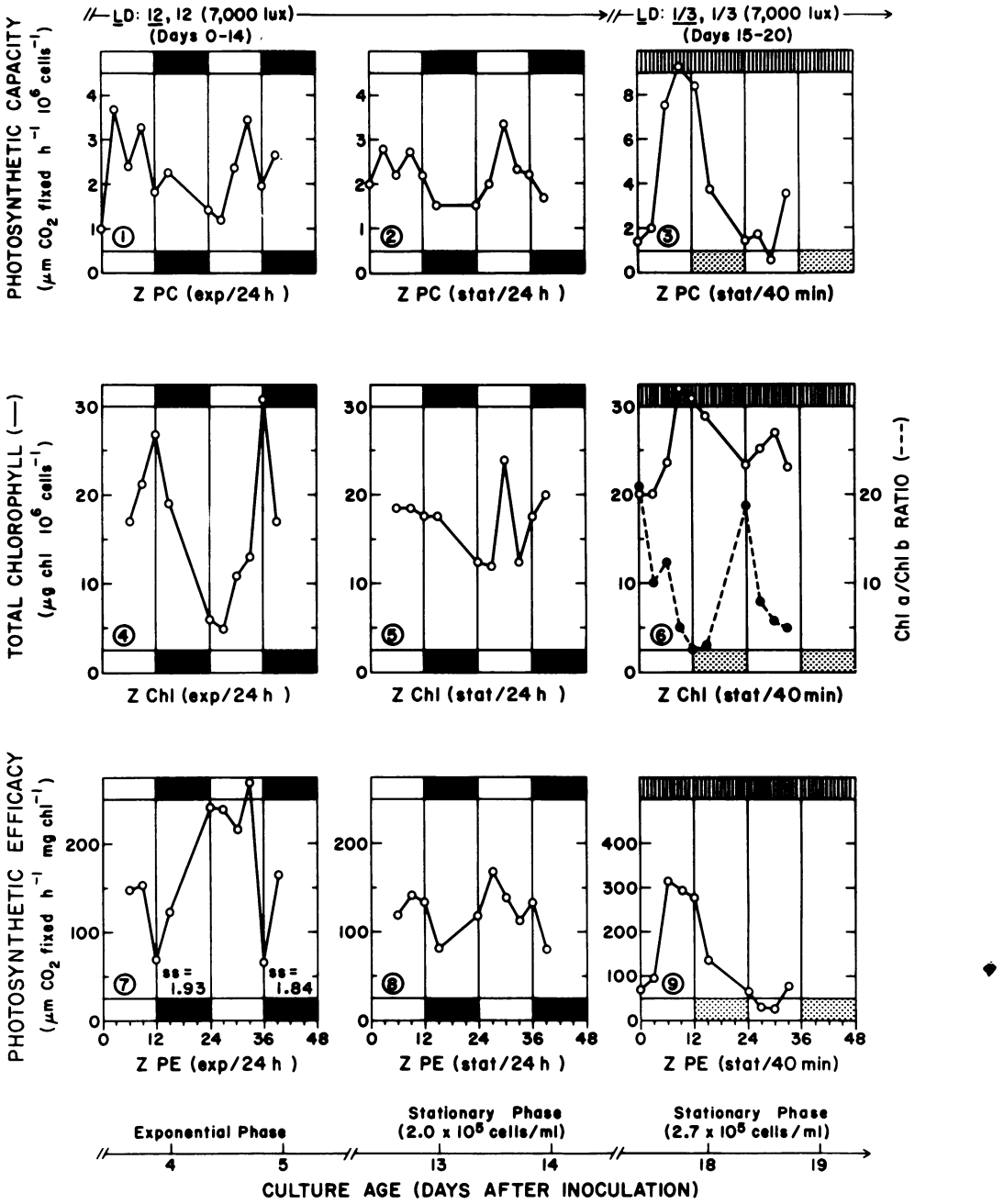

FIG. 2. Photosynthetic rhythms in photoautotrophically batch-cultured *E. gracilis* (Z). PC (panels 1–3, top row), total Chl (panels 4–6, middle row), the Chl *a*/Chl *b* ratio (Chl *a*/Chl *b*: panel 6, - - -), and PE (panels 7–9, bottom row) were monitored at various intervals during a 20-day time span after initial inoculation of the culture and are expressed in the units given on their respective ordinates. Each parameter was measured under three sets of conditions: during the exponential phase of growth across one or more synchronous growth cycles in LD: 12,12 (7,000 lux) (exp/24 h: panels 1, 4, and 7, left column [successive step sizes of 1.93 and 1.84]); during the stationary (infradian, or slowly dividing) phase in LD: 12,12 (7,000 lux) (sta/24 h: panels 2, 5, and 8, middle column [cell concentration = 2.0×10^5 cells ml^{-1}]); and finally, during the stationary phase (cell concentration = 2.7×10^5 cells ml^{-1}) in a high frequency (40 min) LD: 1/3,1/3 (7,000 lux) cycle (stat/40 min: panels 3, 6, and 9, right column). The light regimen imposed is shown on an absolute time scale at the top of the figure; absolute culture age is given at the bottom.

however, were not always as evident. This can be attributed to two major factors: (*a*) the τ_{FR} values ($\bar{\tau} \simeq 30$ h) differed slightly among the five cultures, and though an attempt was made to place all of the normalized curves in register, some variation was inevitable in selecting CT 0 (or the peak value, or acrophase); and (*b*) the degree of division synchrony in these dividing cultures in LD: 1/3,1/3 was less than that routinely observed in LD: 12,12 (11, 12). Thus, the fission "bursts" had a duration as long as 20 h (*i.e.* two-thirds of τ_{FR}) in contrast to the 6 to 8 h typical under an entraining 24-h LD cycle. This reduction in the degree of synchrony has been noted previously for *Euglena* in other types of high frequency regimens (13, 14).

The composite plot for variation in total Chl content and for PE (derived from the data for PC and Chl) in dividing cultures exposed to high frequency LD cycles (Fig. 4, left column) was not so rhythmic as that for PC. This is due not only to the reasons given above, but also to the fact that the well defined rhythms observed in each of the five *individual* experiments exhibiting

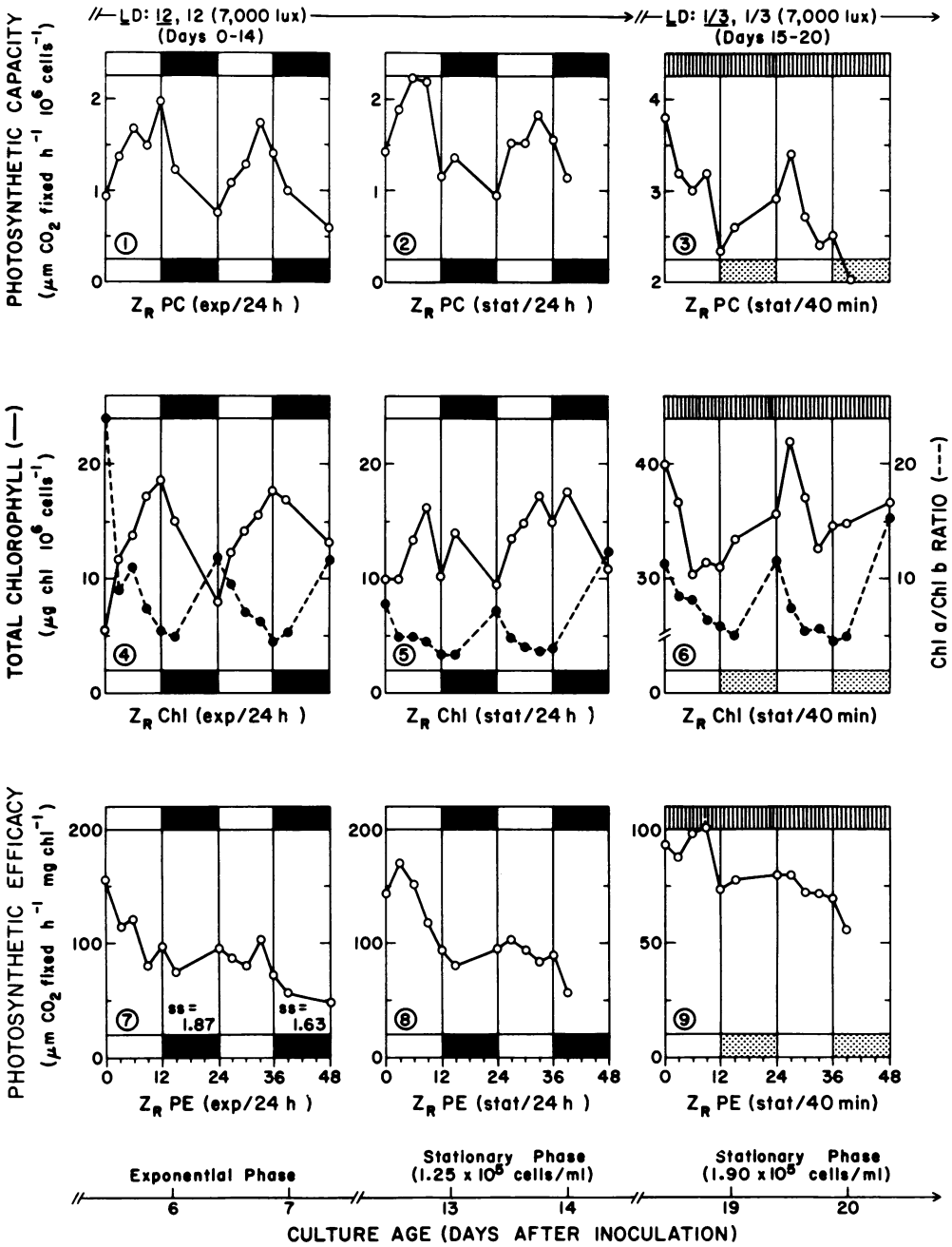

FIG. 3. Photosynthetic rhythms in a photoautotrophically batch-cultured DCMU-resistant strain (Z_R) of *E. gracilis* (Z). The experimental protocol and measurements are the same as those illustrated in Figure 2 for the wild type Z strain. Step sizes for the two monitored synchronized division bursts during the exponential phase of growth (left column) were 1.87 and 1.63. The cell concentration during the stationary phase interval during which measurements were made (middle column) was about 1.25×10^5 cells ml^{-1} in LD: 12,12. The titer had eventually attained 1.90×10^5 cells ml^{-1} when measurements were made in the high frequency LD: 1/3,1/3 cycle (stat/40 min, right column).

varying phase relationships to the concomitant rhythms in PC, whose period and phase were used for normalization. The data for the individual cultures indicated that the overt rhythm in PC was generated by variation in Chl *a*; Chl *b* content varied only slightly and randomly around a mean value of 3 $\mu g/10^6$ cells, whereas Chl *a* content oscillated with a period of about 30 h and ranged between 15 and 30 $\mu g/10^6$ cells. The time at which PC typically showed a maximal value (CT 9–12) corresponded to the time at which PE was also maximal but at which Chl had rarely, if ever, attained maximal concentration.

As the cultures in LD: 1/3,1/3 reached the stationary (infradian) phase where little or no cell division occurred (thus negating the influence of variations in cell cycle duration or of subpopulations of cells), the rhythms in PC and in PE became increasingly better defined (Fig. 4, right column) with large amplitudes and $\bar{\tau}_{FR} \simeq 30$ h (see also Figs. 2 and 3, panels 3 and 9). The variations in Chl in each of the five *individual* experiments were well marked, and although they showed varying phase angles to the corresponding rhythms in PC, the composite statistical plot (Fig. 4) clearly shows a persisting circadian rhythm, albeit necessarily of reduced amplitude.

DISCUSSION

The data obtained in this work document the existence of 24-h

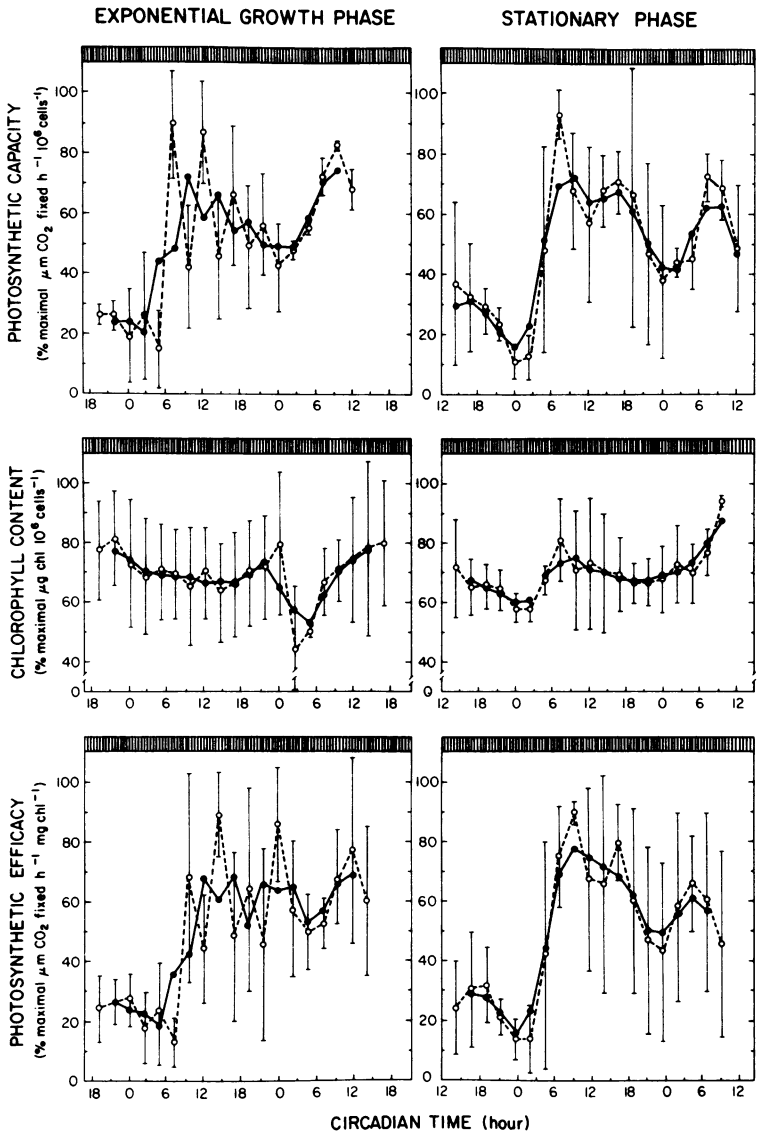

FIG. 4. Statistical analysis of photosynthetic rhythms in photoautotrophically batch-cultured *E. gracilis* (Z) (Stony Brook) in high frequency (40-min) LD: 1/3,1/3 (7,000 lux) cycles. PC, total Chl, and PE were monitored during both the exponential phase of growth (left column) and the stationary (infradian) phase (right column) in five different experiments. For each experiment, data were expressed as per cent of the maximal value. The mean values were then plotted (O- - -O); vertical bars indicate twice the standard errors of these means. The heavy curves (●——●) indicate moving three-point averages of the mean values. Subjective circadian time is given on the abscissa; the free running periods ($\tau_{FR} \simeq 30$ h) were first normalized to 24 h, whereupon circadian time 0 was ascertained by reference to the point (minimal value of the oscillation in most instances) corresponding to the onset of light in the rhythm of PC entrained by LD: 12,12 (Fig. 1A). Absolute values of the different parameters in the individual experiments were in the same range as those given in Figures 1, 2, and 3.

rhythms of photosynthetic CO_2 fixation and of Chl content during both the synchronous growth cycle of *Euglena* and subsequently during the infradian (stationary) phase. The overt rhythms are clearly endogenous and autonomously oscillatory since they not only are manifested independently of the cell cycle itself, but also persist for extended time spans (with a period approximating 30 h) in 40-min (LD: 1/3,1/3) light-dark cycles following initial synchronization in LD: 12,12. Such high frequency regimes, for all intents and purposes, can be formally equated with constant illumination (LL) explored previously for CO_2 fixation (32) and O_2 evolution (20, 21) in infradian cultures of *Euglena*, and have been utilized successfully in the past to elicit free running circadian rhythms of cell division in photoautotrophically cultured *Euglena* having periods of about 27 to 28 h also (13, 14).

In this study, the period (τ_{FR}) of the photosynthetic rhythms under the imposed LD: 12,12 cycle matched that (T) of the driving regime (*i.e.* $\tau \equiv T = 24.0$ h), and thus, the rhythms can be formally described as being entrained, or synchronized. Under the LD: 1/3,1/3 regimen, both dividing and stationary cultures displayed photosynthetic rhythms having significantly longer periods (about 30 h), and hence, can be described as free running. The rhythm of cell division exhibited the same 30-h period length (13, 14). It may be that the high frequency LD cycle does exert some influence on τ_{FR}, just as τ_{FR} is a function of the intensity of illumination under free running conditions in higher organisms.

The present data do not permit us to determine with certainty whether desynchronization among the various free running rhythms occurs due to differences in their τ_{FR} values (32). On the one hand, this appears *not* to be the case on the basis of the close correspondence between the rhythms in PC and Chl for both the Z strain (Fig. 2, panels 3 and 6) and the Z_R strain (Fig. 3, panels 3 and 6) in free running conditions; in each case they continue to bear the same phase angle to each other as they did in the prior entraining LD: 12,12 cycle. (The rhythms in the two strains,

however, did have different phase relationships to the *projected* LD cycle; this probably reflects differences in the initial, transient phase-shifting effects of the LD: 1/3,1/3 cycle, which was imposed at the 11th h or the 3rd h of the 12-h light period for Z and Z_R, respectively.) On the other hand, we noticed that in the *individual* experiments upon which Figure 4 was based, there was a varying phase angle relationship between the rhythms in PC and in Chl in both dividing and in nondividing cultures maintained in LD: 1/3,1/3. If this indeed be the case, it would constitute a first report for desynchronization among free running circadian rhythms in a *unicellular* organism, suggesting the existence of a multioscillator system.

Twenty-four-h rhythms in photosynthetic activities have been routinely observed during the exponential growth phase in synchronously dividing cultures of many algae and higher plants subjected to 24-h LD cycles (16, 31, 32). Our analysis (Fig. 1A) reveals the existence of a maximal peak in PC at the end of the 12-h light period and the secondary peaks at the 3rd h of light and at the middle of the dark period, 6 h after the onset of darkness. During the first 3 h of light 10% of the maximum cellular Chl content has accumulated, but the ability of the cells to fix CO_2 has increased by 25%, generating a corresponding high value for PE (Fig. 1B). Chl continues to accumulate while PE decreases. The results from *individual* experiments on cultures in LD: 1/3,1/3 all indicated that the maximal value for PC never corresponded to the peak in Chl content. These observations suggest that either Chl is playing a different role in the channeling of energy or that the activities of the Calvin cycle enzymes are rhythmically changing. The oscillations in Chl content do not appear to be tightly coupled to the cell division cycle itself even in dividing populations because the factor by which Chl content increased often was considerably greater than the stepsize for the synchronous division burst.

Our data on the variations in PC, PE, Chl content and Chl *a*/Chl *b* ratio during the growth cycle—and, in particular, during the circadian cycle in nondividing cells—differ somewhat from work on other eukaryotic algae such as division-synchronized *Chlorella, Chlamydomonas,* and *Scenedesmus* (28). Although several workers have reported that the diurnal variation in PC observed in natural planktonic populations is a result of a concomitant change in Chl *a* concentration (29, 33), in the majority of cases no close correspondence between PC and Chl content has been found (15, 18, 25, 32). Recent tests for circadian oscillations in pigment content in nondividing cultures under free running conditions of constant illumination have been negative; Prézelin *et al.* (26, 27) found no persistent rhythm in Chl concentration in *Gonyaulax* and other marine dinoflagellates, and Lonergan and Sargent (21) detected no obvious rhythm in either Chl content or Chl *a*/*b* ratio in *Euglena*. (In the latter case, a drop in the *a*/*b* ratio occurred during the light portion of the entraining LD: 10,14 cycle [*cf.* our Fig. 3, panels 4, 5, and 6] and during the 1st day only of the ensuing constant dim illumination.) Nevertheless, a circadian rhythm of net photosynthesis and of Chl content has been shown in *Chenopodium* (2), a nonalgal species.

We suggest that several interrelated factors could be responsible for the rhythm in the capacity of the cell to fix CO_2 photosynthetically: variations in the amount of Chl per cell (both in dividing and in nondividing populations), variations in the organization and distribution of the Chl modulating their activities in the photosynthetic units (PSUs) within the thylakoids, and rhythmic changes in the activities of the Calvin cycle enzymes.

In lactate-synchronized, dividing populations of *Euglena*, analysis by means of fluorescence-induction curves (9, 17) showed no changes in PSII units during the cell cycle (8). Under such conditions, lactate inhibits photosynthetic activity (1). The circadian rhythm of O_2 evolution may be correlated with membrane alterations in nondividing, autotrophic cultures of *Euglena*. On the basis of their investigation of the circadian rhythm of photosynthesis in *Gonyaulax*, Prézelin and Sweeney (27) suggested that an uncoupling site of the PSU may occur between the reaction center and the light-harvesting pigments; the periodic inactivation of PSUs would generate a rhythm in Chl fluorescence yield that is 180° out of phase with the observed rhythm in PC (as previously reported by Senger and co-workers [28] for other algae). This rhythm might be generated by periodic conformational changes of the thylakoid membrane caused by circadian ion fluxes (24). Such hypotheses all involve intrinsic, topological, rhythmic rearrangements of the PSU components within the photosynthetic membranes. Our data, showing a persisting circadian rhythm in the qualitative and quantitative pigment composition of the *Euglena* cell, also suggest that such changes may play a major role in the generation and modulation of the overt rhythm in photosynthetic CO_2 fixation in both dividing and nondividing populations.

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